In Vivo Nuclear Magnetic Resonance Study of the Osmoregulation of Phosphocholine-Substituted β-1,3;1,6 Cyclic Glucan and Its Associated Carbon Metabolism in *Bradyrhizobium japonicum* USDA 110

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A phosphocholine-substituted β-1,3;1,6 cyclic glucan (PCCG), an unusual cyclic oligosaccharide, has been isolated from *Bradyrhizobium japonicum* USDA 110 (D. B. Rolin, P. E. Pfeffer, S. F. Osman, B. S. Swergold, F. Kappler, and A. J. Benesi, Biochim. Biophys. Acta 1116:215–225, 1992). Data presented here suggest that PCCG synthesis is dependent on the carbon metabolism and that osmotic regulation of its biosynthesis parallels regulation of membrane-derived oligosaccharide biosynthesis observed in *Escherichia coli* (E. P. Kennedy, M. K. Rumley, H. Schulman, and L. M. G. van Golde, J. Biol. Chem. 251:4208–4213, 1976) and *Agrobacterium tumefaciens* (G. A. Cangelosi, G. Martinetti, and E. W. Nester, J. Bacteriol. 172:2172–2174, 1990). Growth of *B. japonicum* USDA 110 cells in the reference medium at relatively low osmotic pressures (LO) (65 mosmol/kg of H_2O) caused a large accumulation of PCCG and unsubstituted β-1,3;1,6 cyclic glucans (CG). Sucrose and polyethylene glycol, nonionic osmotica, reduce all growth rates and inhibit almost completely the production of PCCG at high osmotic pressures (HO) above 650 and 400 mosmol/kg of H_2O), respectively. We used in vivo ^{13}C nuclear magnetic resonance spectroscopy to identify the active osmolytes implicated in the osmoregulation process. The level of α,α-trehalose in *B. japonicum* cells grown in autoclaved or filter-sterilized solutions remained constant in HO (0.3 M sucrose or 250 g of polyethylene glycol 6000 per liter) medium. Significant amounts of glycogen and extracellular polysaccharides were produced only when glucose was present in the autoclaved HO 0.3 M sucrose media. The results of hypo- and hyperosmotic shocking of *B. japonicum* USDA 110 cells were monitored by using in vivo ^{31}P and ^{13}C nuclear magnetic resonance spectroscopy. The first observed osmoregulatory response of glycogen-containing cells undergoing hypoosmotic shock was release of P_i into the medium. Within 7 h, reabsorption of P_i was complete and production of PCCG was initiated. After 12 h, the PCCG content had increased by a factor of 7. Following the same treatment, cells containing little or no glycogen released trehalose and failed to produce PCCG. Thus the production of PCCG/CG in response to hypoosmotic shocking of stationary-phase cells was found to be directly linked to the interconversion of stored glycogen. Hyperosmotic shocking of LO-grown stationary-phase cells with sucrose had no effect on the content of previously synthesized CG/PCCG. The PCCG/CG content and its osmotically induced biosynthesis are discussed in terms of carbon metabolism and a possible role in hypoosmotic adaptation in *B. japonicum* USDA 110.

Large, cell-associated β-1,2 cyclic glucans are commonly found in bacteria of the family *Rhizobiaceae*, which includes *Rhizobium* and *Agrobacterium* spp. *Rhizobium* spp. fix nitrogen in a symbiotic relationship within legume root nodules, and *Agrobacterium* spp. are pathogens causing crown gall tumors on a variety of dicotyledonous plants. The glucans produced by these bacteria are cyclic molecules consisting of approximately 17 to 40 β(1,2)-linked glucose residues (1, 33) that can be modified in various ways such as the anionically substituted forms containing phosphoglycerol (3, 31, 33, 34). The glucans are presumed to be membrane-derived oligosaccharides (MDO), i.e., oligosaccharides produced on the cytoplasmic membrane and released into the periplasmic space (1, 23, 25, 28, 34). With the notable exceptions of *Rhizobium leguminosarum* biovar trifolii TA-1 (9) and *Rhizobium meliloti* GR4 (42), external high osmolarity (HO) generally suppresses the production of cell-associated periplasmic glucans. It is presumed, therefore, that such molecules play a role in the adaptation of these bacteria to low-osmolarity (LO) environments (6, 10, 16, 20, 33). Stock et al. (44) provided evidence in their fundamental study of the periplasmic compartment of *Escherichia coli* and *Salmonella typhimurium* that the periplasm is isoosmotic with the cytoplasm and that a Donnan potential exists across the outer membrane of these bacteria. Dylan et al. (16) confirmed that hypoosmotic adaptation in *R. meliloti* requires β(1,2)-linked glucan. It is generally thought that the relatively large size and cyclic configuration of these molecules allow them to function as fixed osmolytes in the periplasm, thereby permitting a relatively constant periplasm-to-cytosol volume ratio to be maintained (13, 16, 33, 52).

Bradyrhizobium japonicum, in an analogous manner, produces an MDO, a cell-associated branched β-1,3;1,6 cyclic glucan (CG) (32, 41). In addition, we (41) have isolated and characterized a β-1,3;1,6 branched cyclic glucan that contains a zwitterionic phosphocholine group (PCCG). Unlike CG, which occurs as a mixture of four different glucose-containing rings (10 to 13 members), PCCG is a structure with 13 glucose units,

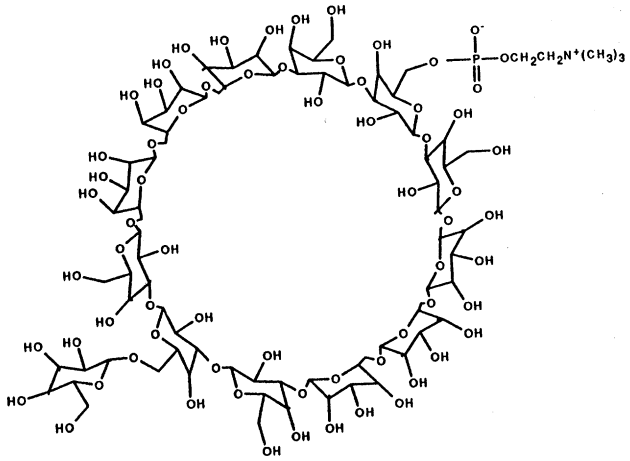

FIG. 1. Structure of PCCG.

1 of which is branched (41), and a phosphocholine group that is linked to a C-6-OH of the 1,3-linked glucose unit within the cycle. The most probable structure for this compound is shown in Fig. 1.

Since phosphatidylcholine is the most abundant phospholipid in *B. japonicum* USDA 110 (35), which is unusual for a gram-negative bacterium, it has been suggested that this phospholipid may have an important role in plant infections (21). From the established pathways for the production of the phosphoglycerol-substituted β-1,2-cyclic glucans as found in *E. coli* (27) and *R. meliloti* (19), we suggest that the phosphocholine head group of PCCG is derived from the turnover of membrane lipids (41). Although evidence seems to indicate that CG is synthesized on the inner membrane (4, 11, 24) and is located in the periplasmic space (30), the precise location of PCCG has not been established.

CG and PCCG were isolated from free-living *B. japonicum* USDA 110 (41) and bacteroids (22, 41) of soybean nodules in the ratios of 1:3 and 1:1, respectively, suggesting that the higher osmotic environment of the nodule might be responsible for suppressed synthesis of PCCG relative to CG.

The production of cell-associated CG is affected by the external osmolarity (30). The accumulation of CG in the periplasm is inhibited in the presence of high concentrations of nonionic osmotica such as sucrose or fructose (30, 49). However, other functional properties of CG have been suggested by others (5, 20, 39, 43). Bhagwat et al. (5) have demonstrated that a *B. japonicum* USDA 110 cosmid clone complementing a cyclic β-1,2 glucan biosynthesis (*ndvB*) mutant of *R. meliloti* was able to specify synthesis of CG. The complemented strain was found to be symbiotically active on alfalfa, indicating that CG and β-1,2 cyclic glucans may have similar functions in their respective organisms. In addition, a more recent report (32a) suggests that CG is biologically active in inducing the production of the phytoalexin glyceolin and the Nod factor elicitor daidzein as assessed with a soybean cotyledon bioassay. Presently nothing is known about the regulated production or biological activity of PCCG.

In this study we have examined the dynamic process of PCCG modulation under long- and short-term osmotic transitions in *B. japonicum* USDA 110 by using in vivo nuclear magnetic resonance (NMR) spectroscopy. ^{31}P-NMR was chosen primarily because its high sensitivity (100% natural abundance) allowed quantitation of rapid bioenergetic and intracellular pH changes while continuously and noninvasively monitoring PCCG. ^{13}C-NMR was used primarily to determine the nature and level of organic osmolyte production associated with the osmoregulation process. The conclusions based on these observations are discussed in the context of a role for PCCG in hypoosmotic adaptation and its implication in carbon metabolism.

MATERIALS AND METHODS

Production of *B. japonicum* USDA 110. *B. japonicum* USDA 110 cells were aerobically grown for 4 days, unless otherwise indicated, at 28°C on a rotary shaker in 1 liter of medium (wide-mouth flasks, 2,800-ml capacity) after inoculation with 10 ml of 3-day-old 50-ml precultures. The medium contained (in grams per liter) $CaCl_2$, 0.015; NH_4Cl 0.16; Na_2SO_4, 0.125; Na_2HPO_4, 0.125; $MgSO_4 \cdot 7H_2O$, 0.18 sodium ferric, EDTA, 0.016; *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 1.3; morpholineethanesulfonic acid (MES), 1.1; arabinose, 2; and yeast extract (pH 6.8), 1; and was supplemented or not supplemented with various concentrations of sucrose used as an osmoticum to modify osmotic pressure. Iron was provided in a chelated form to prevent formation of a precipitate after autoclaving. Up to 5.0 g of fresh cells per flask was produced under these conditions. In some instances the media were filter sterilized with a 0.2-μm-pore-size filter to eliminate the production of glucose and fructose due to sucrose hydrolysis during media autoclaving. When polyethylene glycol 6000 (PEG 6000) was used as an osmoticum, the medium was filter sterilized prior to inoculation to prevent PEG 6000 breakdown.

Before NMR experiments, cells were centrifuged out (19,692 × *g*) in 250-ml bottles for 10 min, rinsed in a solution of 1.3 g of HEPES per liter and 1.1 g of MES per liter (pH 6.8) containing the same sucrose concentration as the growth medium, centrifuged again, and then transferred to the NMR tube. To perform ^{13}C-NMR analysis of cells previously grown in a high sucrose concentration, three rinses with the buffer solution without sucrose were necessary. However, to assess the content of released osmolytes as well as intracellular ones by ^{13}C NMR, we performed no rinsing. Also, when PCCG generation was to be examined, no rinsing was performed. Cell growth was monitored by optical density (A_{650}) measurements with a Beckman DU 70 spectrophotometer. Osmotic pressure was measured with a freezing point osmometer (model 5004 m Osmette; Precision Systems Inc., Natick, Mass.).

NMR experiments. In vivo ^{31}P- and ^{13}C-NMR experiments were carried out with a JEOL 9.4T NMR spectrometer operating at 161.7 and 100.5 MHz, respectively. Typically for ^{31}P experiments, 1.3 to 1.6 g (280 mg [dry weight]) of *B. japonicum* USDA 110 cells was placed in a 10-mm NMR tube and the volume was adjusted with 3 ml of D_2O buffer (10 mM MES–HEPES [pH 6.8]). An airlift assembly, as described by Fox et al. (17), was used to supply the cells with O_2 or N_2. Two drops of silicone antifoam was added to each sample to prevent the entrainment of liquid through the gas outlet tube. Proton-decoupled ^{31}P spectra could be obtained within 15 min (5,000 scans) at 21°C with a 30° pulse angle, a rapid repetition time of 0.16 s, and a spectral width of 16,000 Hz (40). To obtain quantitative spectra the recycling time was changed to 4.1 s and the pulse angle was increased to 80°. These quantitative spectra were obtained with 8,000 scans. For time course experiments, 20,000 scans (1-h accumulations) were used since spectral changes were slow and studies were often extended over periods of 18 to 20 h. A reference capillary (outer diameter, 1.8 mm) containing 0.120 M hexamethylphosphor-

amide (HMPA) was used as a shift and concentration reference for quantitating all compounds. HMPA was assigned a shift position of 30.73 ppm relative to 85% H_3PO_4 (0.0 ppm). The intracellular pH of the organisms was estimated from the cytoplasmic P_i shift and referenced to a standard curve of P_i shift versus pH as described previously (37, 38). Each resonance in the spectrum was identified from extracts which were "spiked" with authentic samples (40). When examining PCCG contents of the cells, the LO-grown cells were placed in buffer with no osmolyte present (17 mosm/kg of H_2O), while HO-grown cells were examined in buffer containing the level of osmolyte used in their growth medium. When the PCCG levels from cells grown for different times and under different osmotic conditions were compared, the PCCG resonance areas were referenced to the HMPA peak area. Each peak was cut out and weighed. The relative dry weight of each sample was used to normalize the area of one independent spectrum to another. Each experiment was replicated a minimum of three times. Prior to an osmotic-shock experiment, either a ^{31}P or a ^{13}C spectrum was acquired to evaluate the state of the cells.

In some experiments the HO (650 mosmol/kg of H_2O)-grown cells were placed in the LO medium (65 mosmol/kg of H_2O) for various times, centrifuged, and resuspended in the NMR buffer solution at pH 6.8 for examination. When examining the PCCG production as a function of different osmotic pressures, cells were isolated by centrifugation and directly placed in the NMR buffer. T1 values for the in vivo metabolites and isolated PCCG were obtained by the inversion-recovery method.

To perform proton-decoupled ^{13}C NMR experiments, approximately 1.5 to 2g (fresh weight) (400 mg [dry weight]) of cells and 1.0 ml of D_2O buffer were placed in a 10-mm NMR tube. Each proton-decoupled, natural-abundance spectrum was obtained at 21 to 22°C, by using bilevel decoupling, a 1.32-s repetition rate, a 70° pulse angle, and a 25,000-Hz spectral width. Most accumulations required 3 h (8,000 scans) for an adequate signal/noise ratio. A 10-Hz exponential broadening factor was applied to all spectra. All shifts were referenced to a spectrum of sucrose (C-2′ anomeric carbon resonance at 104.5 ppm), accumulated with the same spectral parameters used for the in vivo experiments. Each component in the ^{13}C spectra, such as sucrose, trehalose, PCCG, and CG was identified by comparison of the shifts of authentic samples of purified or commercially available compounds.

Transmission electron microscopic methods used for examining *B. japonicum* USDA 110 under different osmotic conditions. (i) Electron microscopy. Cells grown for 4 days in 50 ml of normal incubation medium, with or without 0.3 M sucrose, were used for embedding and thin sectioning. Aliquots of cells in suspension (875 ml) were mixed rapidly in a 1.5-ml tube with 125 ml of 10% glutaraldehyde at room temperature. After standing for 5 min, the cell suspensions were centrifuged and stored at 4°C. Some aliquots of cells in normal and hyperosmotic incubation medium were centrifuged and resuspended in the opposite incubation medium for 60 min before undergoing chemical fixation with glutaraldehyde. Further processing steps were done at room temperature: fixed cell pellets were washed in the matching incubation medium and postfixed in 2% osmium tetroxide solution. After being washed in water, the cell pellets were dehydrated in a graded series of ethanol solutions and embedded in an epoxy resin mixture. Thin sections were cut with diamond knives and stained with uranyl acetate and lead citrate solutions. Observations and photographic images were made with a Philips CM12 electron microscope.

(ii) Image analysis. The contours of the outer and inner membranes of several hundred cross-sectioned cells showing circular or nearly circular profiles in photographic prints at a magnification of ×37,200 were traced onto transparent sheets with an overhead projection pen. Video images of the transparent drawings were digitized, processed to fill the enclosed areas in the drawings, analyzed for areas and roundness, and plotted as frequency histograms by using Imageplus software in an image analysis system (Dapple Microsystems, Sunnyvale, Calif.). The difference between the areas enclosed by the outer and inner membranes was used to identify the size of the periplasmic space.

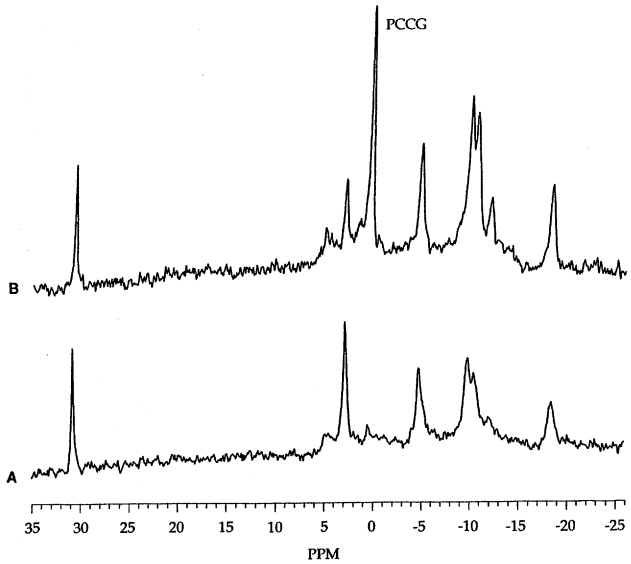

FIG. 2. ^{31}P-NMR spectra (161.7 MHz) of 1.6 g (fresh weight) of *B. japonicum* USDA 110 cells grown for 4 days in medium containing 0.5 M sucrose (650 mosmol/kg of H_2O) (A) and 1.6 g (fresh weight) of *B. japonicum* USDA 110 cells grown for 4 days on medium without added osmoticum (65 mosmol/kg of H_2O) (B).

RESULTS

In vivo NMR of *B. japonicum* USDA 110 cells grown on LO and HO media. *B. japonicum* USDA 110 cells grown for 4 days (stationary phase) in standard medium (65 mosmol/kg of H_2O) produced a very large quantity of PCCG (0.44 ppm), as seen in Fig. 2B. Figure 2B shows a ^{31}P-NMR spectrum of O_2-perfused cells grown under these conditions. The shift of cytoplasmic P_i (3.0 ppm) indicated that the intracellular pH was approximately 7.5. Phosphomonoesters are observed around 5 ppm. The levels of nucleoside triphosphate (NTP) resonances (γ, α, and β at 4.7, 9.8, and 18.2 ppm, respectively) indicated that oxidative phosphorylation was very active since no indication of nucleoside diphosphates (NDP) was observed. The intensity of the PCCG compared with those of other compounds in these spectra was distorted as a result of the high repetition rate used (38). However, quantitatively acquired spectra showed that the ratio of PCCG to NTP was 6.5:1.

To analyze the response to increased medium osmolarity, bacteria were grown in reference medium supplemented with 0.5 M sucrose (650 mosmol/kg of H_2O). These cells produced very little PCCG (Fig. 2A). The cytoplasmic pH was more acidic (pH 7.36), as indicated by the P_i resonance at 2.68 ppm. Phosphomonoesters typically observed at 5.0 ppm were low in

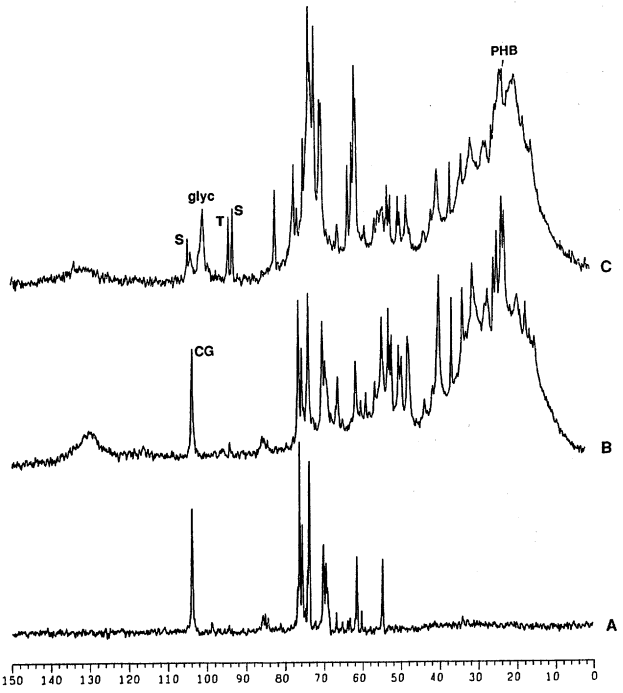

FIG. 3. ^{13}C-NMR spectra (100.4 MHz) of a 60:40 mixture (5 mg) of PCCG and CG isolated from *B. japonicum* USDA 110 cells (10,000 scans) (A), perfused *B. japonicum* USDA 110 cells (400 mg) grown on LO media (65 mosM) (8,000 scans) (B), and *B. japonicum* USDA 110 cells grown on autoclaved HO media (0.3 M sucrose [439 mosmol]), washed twice with D_2O buffer to remove sucrose, and resuspended in buffer (8,000 scans) (C). S, sucrose (residual from medium); T, trehalose; glyc, glycogen.

concentration in these cells. For the same quantity of cells, the overall levels of nucleotides, NTP, UDPG, and NAD was found to be approximately 40% lower in the HO cells and the ratio NTP/P_i was one-third lower.

^{13}C-NMR was used to determine the nature and level of major organic osmolytes associated with the osmoregulation process. The corresponding 100.4-MHz proton-decoupled natural-abundance ^{13}C spectra of HO and LO *B. japonicum* USDA 110 are shown in Fig. 3C and B, respectively, along with the spectrum of a 60:40 mixture of PCCG and CG isolated and purified from *B. japonicum* USDA 110 cells grown under LO conditions (Fig. 3A). In Fig. 3A, resonances characteristic of the β-C-1(1→6) and β-C-1(1→3) were observed at 103.8 ppm and the C-3(1→3), C-6(1→3), $CH_2\underline{C}H_2$-OP, and $CH_2N(\underline{C}H_3)_3$ resonances were observed at 86.0, 62.0, 60.5, and 54.5 ppm, respectively. The additional complex assignments have been described previously (41). Figure 3B, the spectrum of oxygenated cells grown under LO conditions for 4 days, demonstrates the presence of a significant quantity of PCCG and CG in approximately the same proportions as found in the isolated material (Fig. 3A). We also noted the presence of a broad polyhydroxybutyrate (PHB) resonance (2) and lipid resonances from 15 to 35 ppm and amino acid resonances in the range of 20 to 55 ppm. A trace of α,α-trehalose was also evident from the peak at 94.1 ppm (C-1). The broad resonance at approximately 130 ppm represented unsaturated carbons associated with lipids. The corresponding ^{31}P spectrum (see Fig. 8A) indicates that the HO-grown cells produced less than 12% of the PCCG made by cells grown under LO conditions. The spectrum (Fig. 3C) derived from the HO (0.3 M sucrose)-

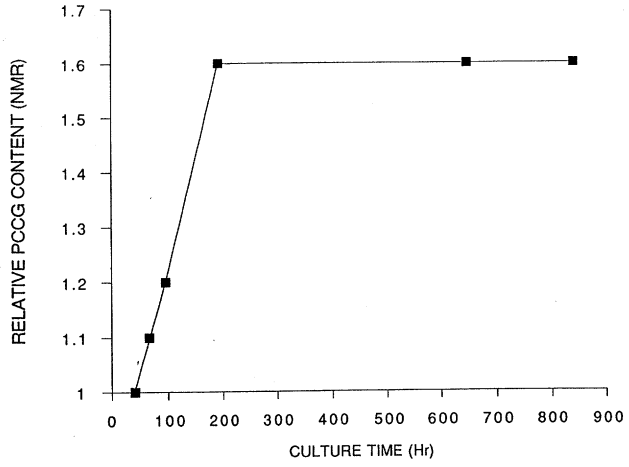

FIG. 4. Production of PCCG as a function of culturing time.

grown cells showed only minor amounts of PCCG and CG. Total glucan production amounted to only 25% (13% CG, 12% PCCG) of that found in LO-grown cells. Residual sucrose remaining in the HO medium after removal of the cells displays resonances at 104.5, 92.9, and 82.2 ppm (C-2, C-1, and C-4, respectively [Fig. 3C]). A sizeable increase in trehalose was detected by its C-1 resonance at 94.1 ppm (Fig. 3C). As in Fig. 3B, PHB, fatty acid, and amino acid resonances were observed upfield; however, considerably less unsaturation was noted at 130 ppm (Fig. 3C). However, most striking was the dominant accumulation of the storage polymer glycogen, characterized by its C-1(1→4) resonances at ca. 100 ppm, C-4 at 77 ppm, C-3 at 76 ppm, C-2 and C-5 at 74 ppm, and C-6(1→4) at 60.8 ppm (29) (Fig. 3C). From the narrowness of its lines, it is apparent that the glycogen within these cells has a high degree of motional freedom. Using peak areas of the ^{31}P-PCCG resonances at 0.44 ppm (Fig. 2), normalized to the external HMPA reference and weights of each sample, we were able to monitor the production of PCCG during its growth. We note from Fig. 4 that PCCG production in LO medium (65 mosmol/kg of H_2O) continued beyond 96 h, the beginning of the stationary phase, to 192 h, after which levels remained constant. Production of PCCG was also examined as a function

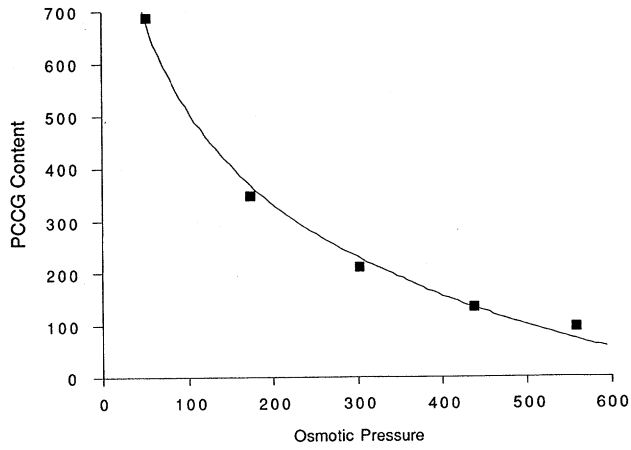

FIG. 5. Production of PCCG as a function of osmotic pressure (in milliosmoles per kilogram of water).

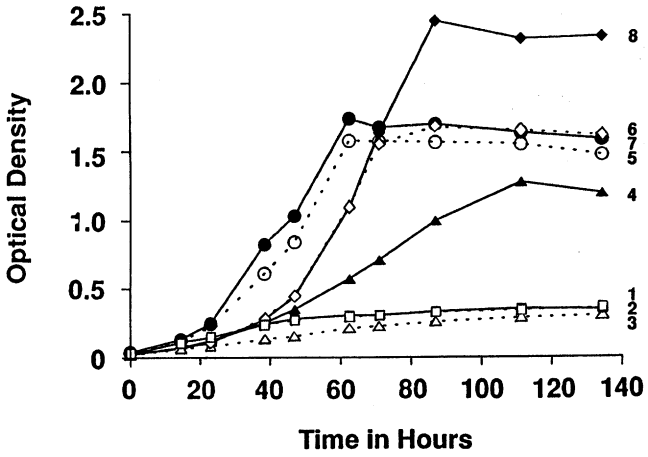

FIG. 6. Growth curves for *B. japonicum* USDA 110 cells grown on media containing as carbon source 1 g of yeast extract per liter (curves 1 and 2); 0.3 M sucrose solution and 1 g of yeast extract per liter (curves 3 and 4); 2 g of arabinose per liter and 1 g of yeast extract per liter (curves 5 and 6); or 0.3 M sucrose solution, 2 g of arabinose per liter, and 1 g of yeast extract per liter (curves 7 and 8). The media were autoclave sterilized at high temperature (curves 1, 3, 5, and 7) or sterilized by filtration (curves 2, 4, 6, and 8). Growth was measured by monitoring the optical density at 650 nm.

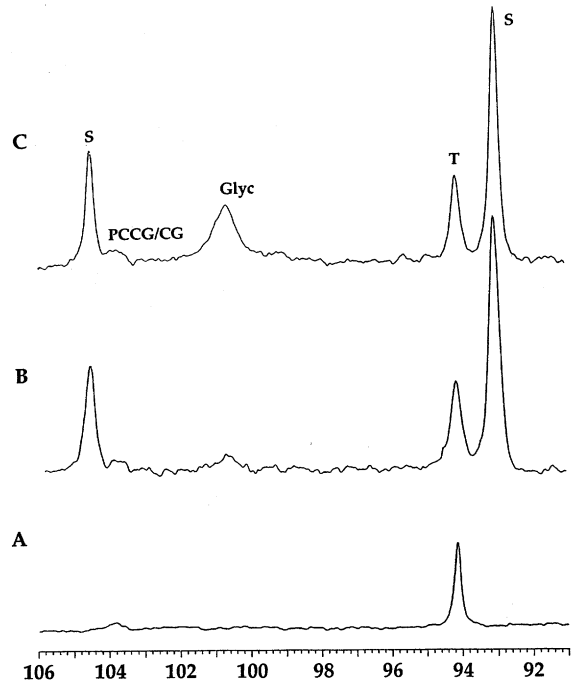

FIG. 7. Low-field region of the 100.4-MHz ^{13}C-NMR spectrum (8,000 scans) of *B. japonicum* USDA 110 cells grown in media containing 250 g of PEG 6000 per liter and 2 g of arabinose per liter, filter sterilized, and grown for 2 weeks (A); 0.3 M sucrose and 2 g of arabinose per liter, filter sterilized, and grown for 4 days (B); and the same ingredients as in panel B but autoclave sterilized (C). S, sucrose (residual from media); Glyc, glycogen; T, trehalose.

of osmotic pressure with sucrose as the osmolyte. A monotonic decline in PCCG content with increasing sucrose concentration is apparent in Fig. 5. Beyond 550 mosmol/kg of H_2O, the PCCG content leveled off to approximately 12% of the maximum amount we observed at 65 mosmol/kg of H_2O, of which the latter represents approximately 0.6% of the dry weight of the cells.

Effects of autoclaving HO 0.3 M sucrose media on the growth of *B. japonicum* USDA 110 cells and production of glycogen. Bacteria were grown for various times, with differing amounts of carbon sources (yeast extract, arabinose, glucose) and osmolarities, using sucrose or PEG 6000 as the nonionic osmoticum. Figure 6 illustrates the effects of various carbon sources and different osmotic pressures on the growth of *B. japonicum* USDA 110 cells. With no added carbon sources apart from the yeast extract (autoclaved or filtered [Fig. 6, curves 1 and 2, respectively]), the bacterial growth was very limited, as was the growth in medium supplemented with filter-sterilized 0.3 M sucrose (curve 3). However, the same medium used to obtain curves 1 and 2 but autoclaved (curve 4) showed additional cell growth, providing indirect evidence of sucrose hydrolysis and production of glucose during the high-temperature sterilization. ^{13}C NMR analysis demonstrated that autoclaved 0.3 M sucrose solutions used to grow *B. japonicum* USDA 110 cells contained approximately 0.8 g of glucose and fructose per liter as a result of the high-temperature sterilization (data not shown). These sugars were not detectable in filter-sterilized (pore size, 0.2 μm) solutions of the same medium.

The maximum growth rate of *B. japonicum* USDA 110 was obtained with media containing yeast extract with arabinose added as the carbon source, either filtered or autoclaved (Fig. 6, curves 5 and 6). Addition of filtered or autoclaved 0.3 M sucrose (curves 7 and 8) to the medium inhibited the growth during the first 45 h. Following this adaptation period, the growth rate was restored. For the autoclaved medium containing approximately 0.8 g of glucose and fructose per liter, additional growth was observed (curve 8).

Figure 7 shows the low-field region of the ^{13}C-NMR spectra of the *B. japonicum* USDA 110 cells grown under HO conditions. We show that cells grown in the autoclaved, sterilized medium containing 0.3 M sucrose produced a significant amount of glycogen (100.8 ppm) in addition to trehalose (94.1 ppm) (Fig. 7C). The same result was also obtained when the cells were grown on filter-sterilized 0.3 M sucrose solutions containing yeast extract, the same amount of arabinose as above, and an additional 1 g of glucose, whereas cells grown in media containing (filter-sterilized) 0.3 M sucrose (Fig. 7B) or 250 g of PEG 6000 per liter (Fig. 7A) as the osmoticum produced little or no glycogen. All four treatments produced cells with negligible amounts of PCCG or CG, as evidenced by the absence of the cyclic glucan C-1 resonance at 103.8 ppm. To verify that glucose was responsible for the production of glycogen, we grew cells on glucose as the only carbon source under LO conditions. Under these conditions, the cyclic glucan mixture (PCCG, CG) and glycogen were produced in equal amounts (data not shown).

In vivo ^{31}P studies of *B. japonicum* USDA 110 responses to osmotic shock. Hypoosmotic shocking of *B. japonicum* USDA 110 cells grown in HO medium (650 mosmol/kg of H_2O) was examined to evaluate the possible mechanism for the production of PCCG. *B. japonicum* USDA 110 cells which were centrifuged from media containing 0.5 M sucrose were washed and resuspended in buffer of pH 6.8 (17 mosmol/kg of H_2O). Figure 8 shows the ^{31}P-NMR spectra before (Fig. 8A) and after (Fig. 8B) the transfer from HO to LO conditions. Cells following 4 days of HO growth do not accumulate PCCG (Fig. 8A). Cytoplasmic P_i has a pH of 7.36. After approximately 20 min in the LO medium, we observed two P_i resonances

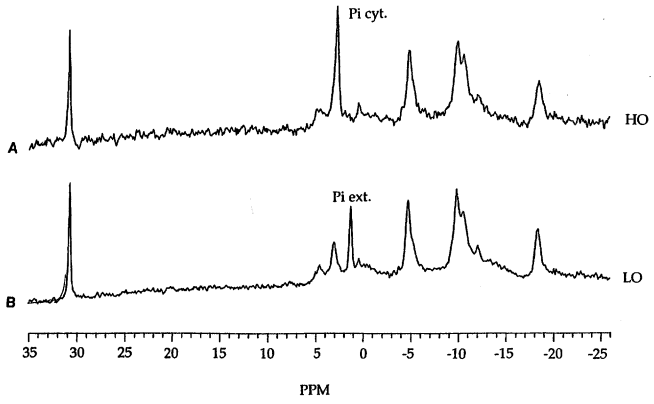

FIG. 8. ^{31}P-NMR spectra (161.7 MHz) of *B. japonicum* USDA 110 cells grown on HO media (0.5 M sucrose, 650 mosmol/kg of H_2O) (A) and the same cells 20 min after transfer to LO buffer (17 mosmol/kg of H_2O) (B).

(approximately 50:50) (Fig. 8B), corresponding to cytoplasmic P_i (pH 7.36) and a more acidic P_i (pH 6.8), respectively. The shift for the low-pH resonance (pH 6.8) matched the medium pH (as confirmed for P_i in LO medium). Removal of the cells demonstrated that approximately 50% of the intracellular P_i had been released into the medium or, alternatively, 50% of the cells released all of their cytoplasmic P_i (data not shown). Following this first step of the osmoregulation process (efflux of P_i), the external P_i began to be reabsorbed by the cells. A ^{31}P NMR time course experiment (12 h) in which the cells were continually oxygenated in LO buffer (17 mosmol/kg of H_2O) is shown in Fig. 9. After approximately 6 h, P_i uptake was complete. However, we did not observe a corresponding increase in the cytoplasmic P_i resonance at ca. 3.0 ppm; rather, we saw the buildup of PCCG, NTP, and NAD resonances. Throughout this period, the phosphomonoester content did not change appreciably, nor was there any significant change in the cytoplasmic pH. The doubling time was approximately 3 h, with a formation constant, k, of 3.21×10^{-3} min^{-1}. The PCCG content increased by a factor of 7 over this 12-h period. In contrast, hypoosmotically shocked *B. japonicum* USDA 110 cells grown in filtered 0.3 M sucrose or 250-g/liter PEG 6000 HO medium produced no PCCG and little or no glycogen (Fig. 7A and B). Also, washing the cells thoroughly with LO medium removed extracellular P_i and trehalose and inhibited the production of PCCG after they were placed in the LO medium in the NMR tube.

Hyperosmotic shocking of cells grown in LO medium (65 mosmol/kg of H_2O) was also investigated. A ^{31}P-NMR time course experiment over a period of 20 h in 0.5 M sucrose buffer demonstrated that PCCG is not degraded following the switch from LO to HO conditions (data not shown). We did observe that the spread of the pH of the cells had narrowed with time and had changed from 6.8 (following osmotic shock) to 7.5, the initial cytoplasmic pH being 7.5 prior to hyperosmotic shocking. ^{31}P- and ^{13}C-NMR spectroscopy was used to evaluate, in vivo, some other aspects of the osmoregulation process. ^{31}P-NMR time course experiments over a period of 20 h in 0.5 M sucrose buffer showed that the level of nucleotides decreased (Fig. 9; resonances of γ, α, and β at 4.7, 9.8, and 18.2 ppm, respectively). At the same time, a ^{13}C-NMR spectrum of these cells showed that the α,α-trehalose content increased by a factor of 4.4 (on a mole basis [data not shown]). Only trace amounts of this disaccharide were detectable prior to the

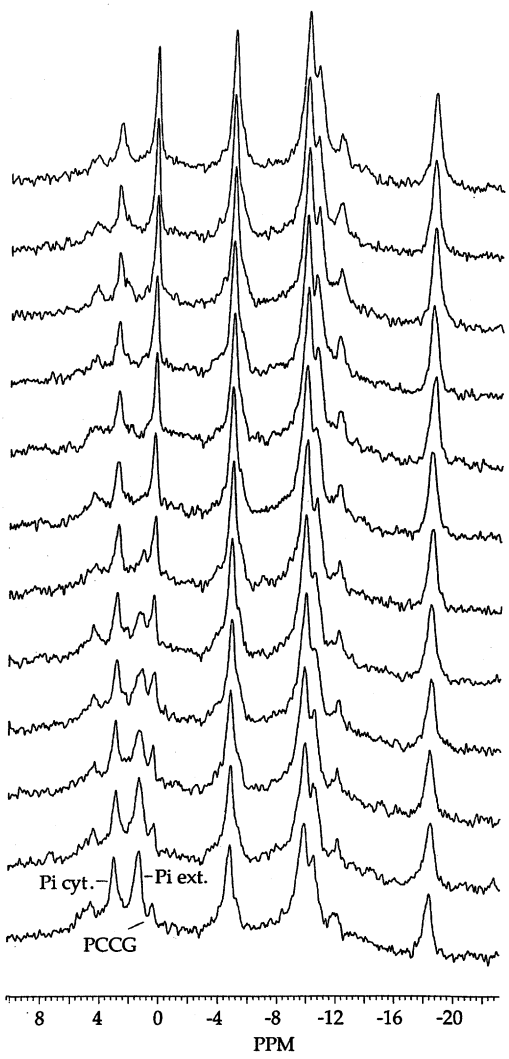

FIG. 9. ^{31}P-NMR time course study (161.7 MHz) of *B. japonicum* USDA 110 cells that had been transferred from HO media (650 mosmol) to LO media (17-mosmol buffer) and perfused with O_2 for 12 h. Each spectrum consists of 20,000 scans (1 h each). The first spectrum is at the bottom of the page.

hyperosmotic shocking. CG and PCCG did not seem to be affected by hyperosmotic conditions over a 20-h period.

Electron microscopy examination of *B. japonicum* USDA 110 cells under different growth and shocking conditions. The cells shown in Fig. 10A grown on HO medium are larger in overall dimensions than are the LO-grown cells in Fig. 10B. The former have proportionally more cytoplasm, which makes their periplasmic space somewhat smaller (ca. 5%) than that of the LO-grown cells (Table 1). The LO-grown cells have periplasmic compartments comparable in size to those reported for *E. coli* and *S. typhimurium* (44). When the cells were hypoosmotically shocked, the cytoplasm expanded, causing a complete contraction of the periplasmic space (Fig. 10C). In contrast, hyperosmotic shocking induced severe shrinkage of the cytoplasm (Fig. 10D). Table 1 shows the changes in the dimensions of the cytoplasm and periplasmic space of *B. japonicum* USDA 110 cells following growth and shocking under different conditions.

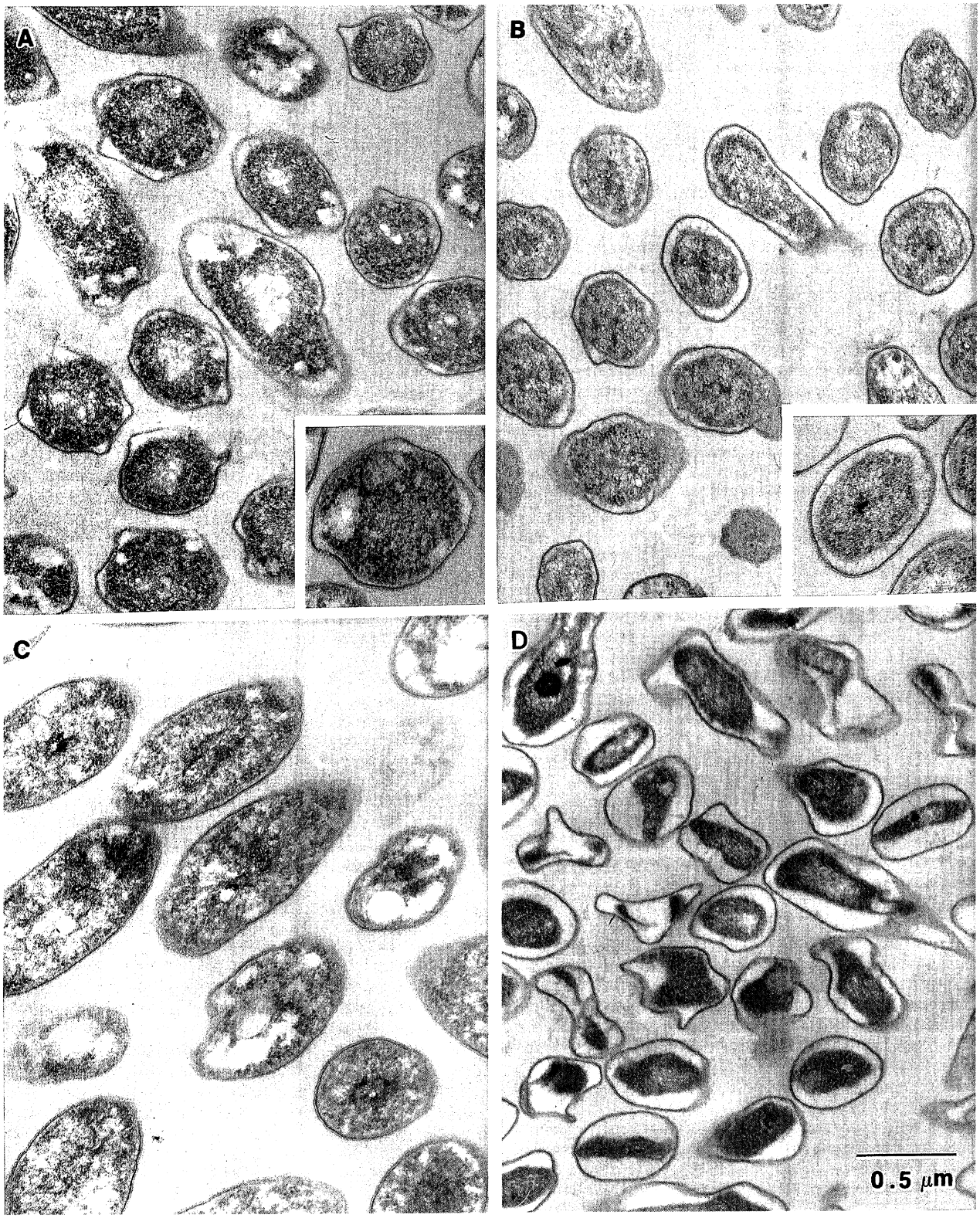

FIG. 10. Transmission electron micrographs of thin sections of *B. japonicum* USDA 110 cells grown in media containing 0.3 M sucrose (A); medium without added sucrose (note the larger periplasmic space [P] and smaller cell size) (B); osmotically shocked, shifted from 650 to 65 mosmol/kg of H_2O (note the smaller periplasmic spaces) (C); and osmotically shocked from 65 to 650 mosmol/kg of H_2O (note the enlarged periplasmic space and condensed cytoplasm) (D). Magnification of insets, ×60,000.

TABLE 1. Analysis of *B. japonicum* USDA 110 cross-sectional area

Conditions	Area (μm^2)			% of total occupied by periplasm
	Total	Inner membrane	Periplasm	
LO grown	0.139 ± 0.032	0.092 ± 0.024	0.047	33.8
Hyperosmotic shock	0.121 ± 0.015	0.057 ± 0.012	0.064	52.8
HO grown	0.249 ± 0.049	0.173 ± 0.036	0.067	27.9
Hypoosmotic shock	0.255 ± 0.020	0.217 ± 0.026	0.038	14.9

DISCUSSION

Cells grown under HO and LO conditions. Data presented here suggest that the PCCG and CG synthesis is dependent on the carbon metabolism and that regulation of this biosynthesis is comparable to the osmotic regulation of MDO biosynthesis observed in *E. coli* and *Agrobacterium tumefaciens* (18, 27, 52). The need to adjust to external osmotic changes is one of the fundamental challenges faced by all cells. Most organisms are unable to control the extracellular environment and therefore must adapt to whatever conditions they encounter. Symbiotic gram-negative bacteria such as *B. japonicum* USDA 110 must survive in LO and HO environments in the free-living state in soil and in the HO (200 to 300 mosmol/kg of H_2O) surroundings within the cytosol of the host plant cell. In choosing an osmoticum to study the osmotic response of *B. japonicum* USDA 110, we concluded that sucrose seemed to be quite appropriate since it is the most abundant of all small molecules found within the cell cytosol, the milieu of *B. japonicum* USDA 110 bacteroids (47).

To obtain a better understanding of the metabolic state of *B. japonicum* USDA 110, we examined its bioenergetic status by ^{31}P-NMR and examined the major carbon compounds accumulated by ^{13}C-NMR profiles of 3- to 4-day-old stationary-phase cells grown under different osmotic conditions. From the data presented, we observed that *B. japonicum* USDA 110 cells produced significant amounts of PCCG when grown under relatively LO conditions (65 mosmol/kg of H_2O). These observations appear to parallel those observed for CG (30, 49), as well as those described for β(1,2)-linked glucans (16, 27, 33). It is generally acknowledged that β(1,2)-linked glucans play a major role in hypoosmotic adaptation. These two types of glucans are cyclic and have relatively large cavities. It is thought that β(1,2)-linked glucans, because of their size and perhaps their water-binding ability, may function as a fixed osmolyte in the periplasm (13, 14). Thus they can increase the turgor pressure against the cytoplasmic membrane and thereby help maintain a relatively constant periplasm-to-cytosol volume ratio in active cells. Because the PCCG signal is 6.5 times larger than the cytoplasmic NTP signal (whose concentration can be as high as 5 mM) (26), we anticipate that the total cyclic glucan concentration (CG, PCCG) in the periplasm of LO-grown cells (the periplasm has a volume one-third that of the cytoplasm) could be 196 mM. Although this may be a bit low relative to the osmotic pressure of cytoplasm (200 to 300 mM) (14), it is not uncommon to find oligosaccharides that generate osmotic pressures greater than the value predicted from their concentration (36).

When we monitored the production of PCCG and CG during the growth period, we were able to show that this production does not continue beyond 196 h. The PCCG and CG biosynthesis appears to be related to the growth phase when the cells exhibit an active metabolism. These results agree with the observations of Geiger et al. (19) demonstrating that in stationary-phase cells, the production of phosphoglycerol β-1,2 cyclic glucans was greatly reduced. We presume that in *B. japonicum* USDA 110 cells the same is true, i.e., that transfer of phosphocholine residues from slowly synthesized phosphatidylcholine in the stationary-phase cells inhibits PCCG production because of the accumulation of excess diacylglycerol (19).

When the cells were grown for a long period under the HO conditions (0.3 to 0.5 M sucrose), the production of PCCG and CG was strongly inhibited (by 89 and 86%, respectively). The processes of cyclization and PC substitution, which occur sequentially, both appear to be under osmotic control. Breedveld et al. (7) have also noted a similar drop in the production of glycerol 1-phosphate-substituted β-1,2 cyclic glucans and the unsubstituted cyclic glucan under HO conditions. From Fig. 5 we observe that the production of PCCG does not appear to show a linear relationship with osmotic pressure. However, the total amount of PCCG produced does not accurately describe a change in intracellular or periplasmic PCCG concentrations but, rather, describes the total amount of PCCG associated with cell mass in the NMR coil. Therefore, if PCCG is periplasmic we might expect its periplasmic concentration to be slightly higher in the HO medium since the periplasm is somewhat more contracted under these conditions. Accordingly, this contraction can account for an underestimation of the PCCG concentration in the cells grown under HO conditions and the nonlinearity of the measured values.

In vivo ^{13}C-NMR was used to identify the active osmolytes involved in the hyperosmoregulation process. Trehalose was found in abundance in the HO-grown cells. This organic osmolyte is well recognized in bacteria for its role in osmoprotection through its ability to maintain low water activity in the cytoplasm (8, 13, 46, 50) as well as for its activity in stabilizing organisms against dehydration (12). Little or no trehalose was found in LO-produced cells (Fig. 3), supporting its osmoregulation role. PHB, a well-known carbon storage polymer (2) observed in the ^{13}C spectra of the cells, is not influenced by osmotic conditions. Its role as a carbon and energy reserve (2) does not appear to be involved with PCCG and CG production. This observation is probably related to the fact that PHB does not share any common pathways with PCCG and CG. It is presumed that PHB is significantly degraded only under extreme starvation conditions (8, 51). What is particularly surprising about the cells grown under HO conditions is the predominant production of glycogen (Fig. 3). The relatively narrow peaks of the characteristic glycogen resonances indicate that this biopolymer is made up of relatively short-chain mobile chains (29). Normally, large quantities of glycogen are found in bacterial cells (51) and fungi (29) grown on a rich carbon source. Initially it was not clear why these organisms produced a storage polymer such as glycogen when they were grown on arabinose (2 g/liter) as a main source of C, under HO conditions (0.3 M sucrose). However, we have demonstrated that *B. japonicum* cells grown in autoclaved arabinose media containing 0.3 M sucrose exhibited relatively enhanced growth over a period of days (Fig. 6, curve 8) compared with the filter-sterilized equivalent media (Fig. 6, curve 7). This additional growth was directly attributable to the presence of glucose (fructose is not utilized by *B. japonicum* USDA 110) (49) produced from sucrose hydrolysis during high-temperature sterilization. From these findings it was evident that sucrose is not used as a carbon source by *B. japonicum* USDA 110 cells (45, 48), whereas glucose can be preferentially used to synthesize glycogen, a glucose storage polymer under HO conditions.

Hypo- and hyperosmotic shocking of *B. japonicum* USDA 110 cells. In this study we have used in vivo NMR to examine

the effects of hypo- and hyperosmotic shocking on the dynamic process of PCCG and CG production in stationary-phase cells over relatively short periods. Hypoosmotically shocked cells grown in autoclaved HO medium (0.3 M sucrose) undergo a net flow of water into their cytosol, leading to an increase in turgor pressure. This assumption is confirmed by the comparison of micrographs of cells grown under HO conditions (Fig. 10A) with micrographs of cells shocked from HO to LO conditions (Fig. 10C). The increase in osmotic pressure in the cytoplasm tends to act as a driving force for expansion of the inner membrane with an increase in cell volume, as well as a contraction of the periplasmic space (Fig. 10C). It has been reported that adjustment to a hypoosmotic environment in *E. coli* involved an efflux of potassium (15). Our in vivo ^{31}P-NMR experiments show for the first time that there is also an efflux of cytoplasmic P_i to the external medium (Fig. 8B). At low osmolarity, P_i efflux, among other things, helps to alleviate some of the buildup in turgor pressure; however, this release probably has only a temporary effect on cytoplasmic osmolarity. Our findings confirm that the first step of hypoosmoregulation in bacteria is the release of ions such as K^+ and P_i and the organic-compatible osmoprotectant trehalose.

The adaptation to osmotic downshifts seems to proceed by a biphasic pattern, with an initial P_i efflux followed by the uptake of P_i and synthesis of PCCG and CG (Fig. 9). It is interesting that a similar biphasic pattern was found when *E. coli* was exposed to HO conditions. Dinnbier et al. (15) have shown that the K^+ which is rapidly taken up from the medium is subsequently replaced by trehalose. PCCG and CG, because of their size and perhaps because of their cyclic configuration, could function as fixed osmolytes in the periplasm. Therefore, by increasing the turgor pressure against the cytoplasmic membrane, the cyclic glucans permit a relatively constant periplasm-to-cytosol volume ratio to be maintained (Fig. 10). Dylan et al. (16), by using *ndv* mutants of *R. meliloti*, showed that the lack of glucan under LO conditions resulted in a shrinkage of the periplasmic compartment relative to the cytosol as a result of excessive expansion of the inner membrane and that this imbalance adversely affected the integrity of the cell envelope. For the HO-grown cells to generate PCCG and CG in the stationary phase when challenged with hypoosmotic shock, they must contain a glucose storage polymer (such as glycogen or trehalose) that can be readily converted to CG and PCCG. If the cells have been grown on a medium devoid of glucose, such as filter-sterilized 0.3 M sucrose or PEG 6000, they produce trehalose exclusively (Fig. 7). Upon hypoosmotic shocking, this trehalose is released, leaving the cells deficient in carbon and unable to synthesize the CG.

In contrast to the hypoosmotic shock events experienced by *B. japonicum* USDA 110 cells, little change is observed in the ^{31}P-NMR spectrum of the cells undergoing hyperosmotic shock. Switching the cells from LO to HO does not affect the cell-associated PCCG content. The PCCG synthesized under hypoosmotic conditions is not readily broken down. However, relatively large amounts of trehalose are produced. These results indicate that PCCG and CG may not be suitable sources of carbon for the production of the osmoprotectant trehalose. Alternatively, this fact may also lend weight to the contention that the cyclic glucans are biochemically isolated in the periplasm. It has been established that CG is produced on the plasma membrane (4, 11, 28); however, it is not known whether the synthesizing enzymes are located on the cytoplasmic or periplasmic side of this membrane. Kennedy et al. (28) have proposed that MDO are synthesized and released from the periplasmic side of the plasma membrane. Since PCCG contains the head group (PC) most prevalent in the membrane lipids of *B. japonicum* USDA 110 (35), we suspect that PCCG is an MDO and that, by analogy with MDO phosphoglycerol-substituted β-1,2-cyclic glucans, it is most probably located in the periplasmic space under specified growth conditions (33).

In the case of hyperosmotically shocked cells, the increase in external osmolarity produced by a nonionic osmoticum must initially cause a net flow of water from the cytosol. The severe shrinking temporarily increases the osmotic pressure in the cytoplasm. This assumption is confirmed by a comparison of the micrographs of cells grown under LO conditions (Fig. 10B) with micrographs of cells shocked from HO to LO conditions (Fig. 10D). A gradual increase in cytoplasmic pH is observed over a period of 18 h as a result of the exchange of protons for ions (especially K^+) (13, 50). Many investigations during the last decade have shown that only a small number of low-molecular-weight water-soluble molecules, in addition to K^+, are important in enabling bacterial cells to cope with elevated osmotic pressure (13, 14). The in vivo ^{13}C-NMR spectra of the hyperosmotically shocked *B. japonicum* USDA 110 cells demonstrate that trehalose is indeed a major cytosolic osmolyte generated under these conditions.

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